transporter. Perhaps these pathways are used when bacteria are exposed to the complex molecular content of intracellular organelles of host cells or potentially nutrient-rich biofilms. *Legionella* is reportedly auxotrophic for several amino acids, so it was surprising to find potential genes for their synthetic pathways: cysteine from pyruvate or serine, methionine from cysteine, and both phenylalanine and tyrosine from phosphoenolpyruvate. Even if some of these genes are not expressed under laboratory growth conditions, their presence presumably relates to the organism's ability to persist in diverse environments.

In addition to previously recognized virulence factors (table S5), we identified new candidates (table S6), including homologs of genes encoding virulence functions in other bacteria. Moreover, ~145 apparent secreted or membrane proteases and other hydrolases, some of which may function as virulence factors, exist in *L. pneumophila*. Among fully sequenced organisms, this is exceeded only by the predatory *Bdellovibrio* (*22*). *L. pneumophila* has been proposed to utilize bacterial-induced apoptotic (early) and/or necrotic pore-forming (late) events to exit infected hosts (*23*); its putative hydrolases may be involved in these processes.

The genome sequence of *L. pneumophila* offers the opportunity to explain its broad host range and extraordinary ability to resist eradication in water supplies. Having lists of genes unique to *Legionella* or shared with unrelated bacteria with similar life-styles, it should now be possible to determine experimentally which of them distinguish *Legionella* species displaying different host preferences or pathogenicity.

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Nitric Oxide Represses the *Arabidopsis* Floral Transition

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The correct timing of flowering is essential for plants to maximize reproductive success and is controlled by environmental and endogenous signals. We report that nitric oxide (NO) repressed the floral transition in *Arabidopsis thaliana*. Plants treated with NO, as well as a mutant overproducing NO (*nox1*), flowered late, whereas a mutant producing less NO (*nos1*) flowered early. NO suppressed *CONSTANS* and *GIGANTEA* gene expression and enhanced *FLOWERING LOCUS C* expression, which indicated that NO regulates the photoperiod and autonomous pathways. Because NO is induced by environmental stimuli and constitutively produced, it may integrate both external and internal cues into the floral decision.

The life of flowering plants is divided into two distinct phases, an initial vegetative phase during which meristems produce leaves and a subsequent reproductive phase during which meristems produce flowers. Genetic studies of the timing of flowering in *Arabidopsis* have revealed four major pathways (*1*). The photoperiod and vernalization pathways integrate external signals into the floral decision, whereas the autonomous and gibberellin (GA) pathways act independently of environmental cues (*2*).

NO plays a pivotal role in animals and has emerged as a key growth regulator in plants (*3*, *4*). NO promotes leaf expansion, inhibits maturation and senescence, stimulates light-dependent germination, and promotes deetiolation (*5*, *6*). Excess endogenous NO reduces growth and delays development in tobacco plants (*7*). NO production is induced by biotic and abiotic stimuli, such as drought, salt stress, and pathogen infection (*4*). In addition, substantial NO is emitted from plants into the atmosphere. Conversely, atmospheric NO, a major greenhouse pollutant produced by combustion of fossil fuels, can affect plants. Thus, NO has a central role in coordinating plant growth and development with environmental conditions. However, little is known about the molecular mechanisms underlying the function of NO in plants.

Treatment of *Arabidopsis* seedlings with an NO donor, sodium nitroprusside (SNP), enhanced vegetative growth and delayed flowering (Fig. 1). SNP increased shoot growth by ~65% at low concentrations (≤100 μM), although it inhibited growth at high concentrations (Fig. 1, A and B; fig S1A). The optimal SNP concentration for promoting shoot growth was ~100 μM. A similar promotive effect of

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NO on chlorophyll content was also found (*8*). SNP delayed flowering in a dose-dependent manner, as measured by the increase in rosette leaf number and days to bolting—swift upward growth at the transition to flowering (Fig. 1, A, C, and D; fig. S1A). A standard indicator for flowering time is the number of leaves produced on the primary shoot before the first flower is initiated; plants that flower late form more leaves (*9*).

Exogenously applied NO may not replicate the function of endogenous NO and may have side effects in plants. Thus, analysis of genetic mutants with altered endogenous NO levels was conducted to determine the in vivo relevance of NO. An NO-hypersensitive screen for *NO overproducer* (*nox*) mutants in *Arabidopsis* was performed (*10*). NO inhibition of root growth was used as a phenotype for the initial screen (fig. S1B). Subsequently, NO production was measured with an NO-sensitive dye, 4,5-diaminofluorescein diacetate (*11*, *12*). Six *nox1* alleles were isolated (Fig. 2A; fig. S2) that contained high levels of NO in roots (*8*) and leaves (table S1) compared with wild type (WT) (Fig. 2B). The *nox1* mutant, which refers to *nox1-1* unless otherwise specified, showed the most root-growth hypersensitivity to SNP of all the mutants isolated. Mutants with altered NO biosynthesis or signaling have not yet been isolated via genetic screens, so *nox1* could provide a powerful tool for dissecting NO function. Despite recent identification of two types of NO synthase (NOS), pathogen-inducible iNOS and constitutive AtNOS, the sources of NO in plants remain to be fully elucidated (*4*, *11*, *12*).

NOX1 was identified as either very close or identical to *CUE1* by map-based cloning (fig. S3A). The morphological phenotype of *nox1* was almost identical to that described for *chlorophyll a/b binding protein* (*CAB*) *underexpressed 1* mutant (*cue1*), including small plant size and pale green leaves with a reticulate pattern (*13*). *CUE1* encodes a chloroplast phosphoenolpyruvate/phosphate translocator (*14*). Several lines of molecular genetic evidence demonstrated that *NOX1* is *CUE1* (fig. S3). The *cue1* mutants were hypersensitive to SNP and displayed an elevated

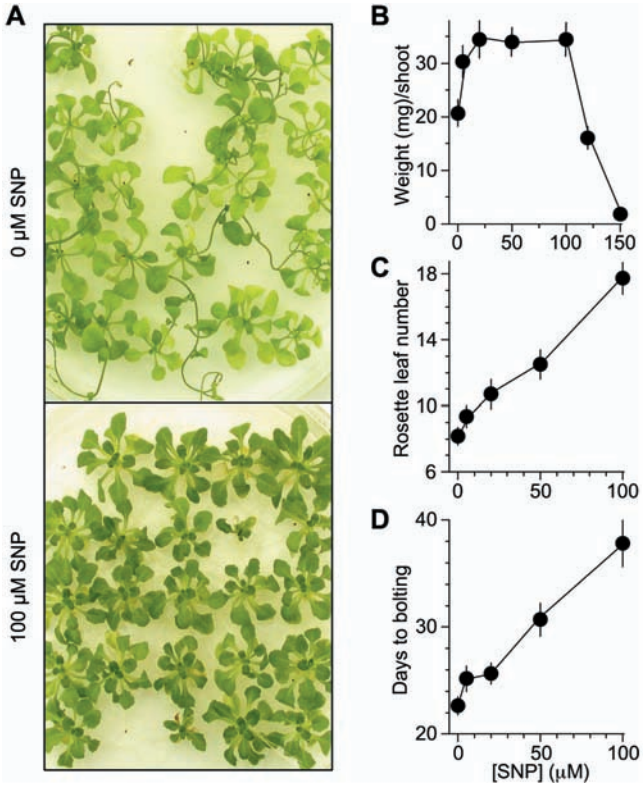

Fig. 1. Exogenous NO promotes vegetative growth but inhibits reproductive development. (**A**) The effects of an NO donor SNP on plant growth and development. *Arabidopsis* seedlings were grown in petri dishes containing SNP during long days (16-hour light/8-hour dark) for 5 weeks (*10*). (**B**) The effect of SNP concentration on shoot growth. (**C** and **D**) The effect of SNP on flowering times. Fresh weight per shoot (B), the rosette leaf number (C), and days to bolting (D) from experiments as in (A) and fig. S1A plotted as a function of the concentrations of SNP that were applied, respectively. Data from four separate experiments are presented (mean ± SD; n = 150 seedlings).

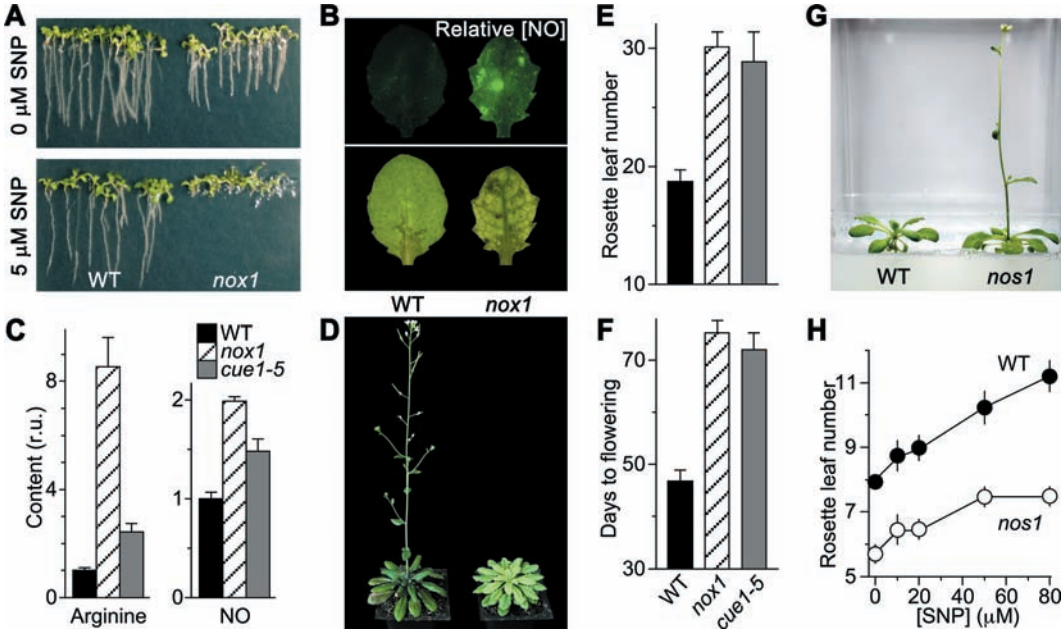

Fig. 2. Endogenous NO represses the floral transition. (**A**) The root growth phenotype in *nox1* mutant. (**B**) The endogenous NO levels in *nox1* and WT. Leaves were stained with DAF-2DA. Fluorescence was analyzed with excitation 490 nm and emission 515 nm (top) with the same exposure times (*10*). White-light images are shown at the bottom. (**C**) The levels of L-Arg and NO in WT, *nox1*, and *cue1-5*. Plants grown under 12-hour light/12-hour dark cycles were harvested 6 hours after dawn (*10*). The absolute levels of L-Arg and NO were 51.8 and 0.45 nmol per gram of fresh rosette leaves in WT, respectively. Values are normalized to those of WT. Each data point represents nine independent measurements (r.u., relative unit). (**D**) The *nox1* mutant flowers late. WT and *nox1* were grown in soil under 12-hour light/12-hour dark cycles and were photographed after 60 days of growth. (**E** and **F**) Flowering times of *nox1* and *cue1-5* mutants. The rosette leaf number (E) and days to flowering (F) from experiments as in (D) were scored (mean ± SD; $n \geq 25$ plants). (**G**) The *NO synthase 1* (*nos1*) mutant that produces fewer NO flowers early under long days. (**H**) The *nos1* mutant flowers earlier than WT under SNP treatments. Experiments similar to that in (G) were analyzed (mean ± SD; n = 20 to 30 plants).

level of endogenous NO. The *cue1* could not complement *nox1* phenotypes. In all six *nox1* alleles, the *CUE1* gene was deleted. Because NO is synthesized from the conversion of L-arginine (L-Arg) to L-citrulline (L-Cit) by NOS (*3*), free L-Arg and L-Cit are several-fold higher in *cue1* mutants than in WT plants (*14*), and *nox1* overproduces NO, we reasoned that disruption of the *CUE1* gene would increase the endogenous L-Arg concentration and thus would promote its conversion to NO. *nox1* and the *cue1-5* mutant harboring a single amino acid mutation (*14*) indeed exhibited larger amounts of L-Arg, L-Cit, and NO than WT (Fig. 2C; fig. S4), indicating that NOS-based NO production occurs in *Arabidopsis*.

Soil-grown *nox1* and *cue1* mutants showed a late-flowering phenotype (Fig. 2, D to F; table S1). This phenotype is not photoperiod-dependent, as *nox1* flowered late under all photoperiods. However, the phenotypic severity was influenced by photoperiods, with 18%, 61%, and 17% increases in the rosette leaf number seen under the light/dark (hours) cycles of 16/8, 12/12, and 8/16, respectively (table S1). This observation is consistent with the light-dependent phenotypes seen in *cue1* mutants (*13*).

In addition, we determined whether down-regulation of endogenous NO promotes the floral transition. The mutant *Atnos1* (*nos1*) that contains a reduced amount of NO (*11*) flowered earlier than WT (Fig. 2G), but still displayed sensitivity to SNP (Fig. 2H). The NO content of *nos1* plants was about 18% that of WT (*8*). The positive correlation between endogenous NO and the number of leaves produced indicates that NO may have a specific role in controlling the floral transition.

To test which components in the floral pathways are affected by NO, we analyzed expression of the floral meristem identity gene *LEAFY* (*LFY*). *LFY* is an important determinant in flower initiation, and its expression increases gradually before flowering commences (*15*). SNP suppressed *LFY* expression in a dose-dependent manner (Fig. 3, A and C). *LFY* expression was low in *nox1*, but was high in *nos1* plants compared with WT (Fig. 3, B and C). The negative correlation between *LFY* expression and endogenous NO suggests that NO repression of the floral transition is mediated, at least in part, by LFY.

Genetic epistasis studies have placed the genes that regulate the floral transition into four major pathways in *Arabidopsis*, all of which converge on the target *LFY* (*2*). The *nox1* mutants flowered late and showed a dwarf phenotype, resembling those of GA-deficient mutants (*16*). GA promoted flowering in *nox1* and WT plants but could not reverse the *nox1* dwarf phenotype (*8*), which suggests that NO may function in parallel with GA. Because *nox1* and mutants of the autonomous pathway flower late on both long and short days (*1*), we reasoned that *nox1* might affect this pathway. The vernalization and autonomous pathways converge on a floral repressor, *FLOWERING LOCUS C* (*FLC*), and late-flowering mutants of the autonomous pathway always have elevated *FLC* expression (*17*). Treatment with low concentrations (≤50 μM) of SNP decreased *FLC* expression, whereas high concentrations (>50 μM) increased *FLC* expression (Fig. 3, D and F). *FLC* expression was high in *nox1* and slightly reduced in *nos1* compared with WT (Fig. 3, E and F). The late-flowering phenotype observed in WT plants treated with high doses of SNP or in *nox1* may result from up-regulation of *FLC* expression. However, the late-flowering phenotype in plants treated with low doses of SNP may be caused by components independent of *FLC*. Nonetheless, the flowering phenotypes observed in *nox1*, WT, and *nos1* are consistent with the expression level of *FLC*, which suggests that endogenous NO may down-regulate the autonomous pathway, which results in late flowering.

Because previous studies have indicated the light-dependent nature of NO effects in plants, and *cue1* mutants show various defects in light perception and photomorphogenesis (*6*, *13*), we investigated whether NO regulates the photoperiod pathway. *Arabidopsis* is a facultative, long-day plant; long days promote flowering (*9*). *CONSTANS* (*CO*) is the most genetically downstream component of this pathway identified so far that promotes floral induction, and it acts as a link between the circadian clock and the control of flowering (*18*, *19*). SNP suppressed *CO* expression in a dose-dependent manner (Fig. 3, G and I). The *CO* expression was high in *nos1* but low in *nox1* plants compared with WT (Fig. 3, H and I). Consistently, *CO* and *FLC* expression were down- and up-regulated, respectively, in *cue1-5* plants (fig. S5). *CO* expression displays a diurnal rhythm (*18*); thus, the repression of *CO* by NO could be due to a reduction of amplitude, a phase shift, or both.

To quantify this effect of NO, we determined *CO* mRNA expression over a 12-hour light/12-hour dark cycle. The overall *CO* mRNA abundance was lower in *nox1* and higher in *nos1* than in WT, although the phase of *CO* mRNA level was not greatly disturbed (Fig. 4, A and B; fig. S6), consistent with previous studies on *gigantea* (*gi*) and *early flowering 3* (*elf3*), mutants of the photoperiod pathway (*20*, *21*). A *gi* lesion

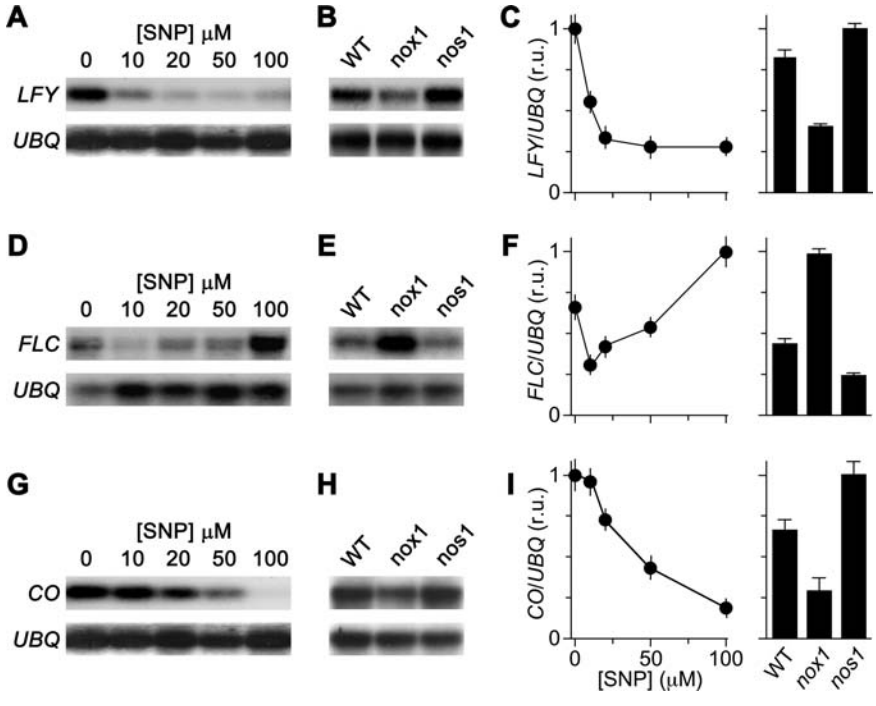

Fig. 3. NO affects the expression of genes that control the floral transition. (**A**, **D** and **G**) The effect of NO on the expression of *LFY*, *FLC*, and *CO*, respectively. Seedlings were grown in media containing SNP under long days. Leaves were collected 8 hours after dawn. The *LFY* and *CO* mRNA abundance was analyzed by using reverse transcription–polymerase chain reaction (PCR) and *FLC* mRNA by Northern blot (*10*). Ubiquitin mRNA (*UBQ*10) was used as a loading control. Similar results were seen for plants grown in 12-hour light/12-hour dark cycles. (**B**, **E**, and **H**) The expression levels of *LFY*, *FLC*, and *CO*, respectively, in WT, *nox1*, and *nos1* plants. Materials were prepared, and mRNA was analyzed as described in (A). (**C**, **F**, and **I**) Analysis of the effects of NO on *LFY*, *FLC*, and *CO* expression, respectively. The relative mRNA abundance was normalized to the *UBQ* levels. The maximum value was arbitrarily set to 1 (mean ± SEM; $n = 3$).

down-regulates *CO* expression, which results in late flowering, whereas an *elf3* lesion up-regulates *CO* expression, which leads to early flowering (*18*). To test whether NO directly affects *CO* expression, we analyzed *GIGANTEA* (*GI*), which is upstream of CO (*21*). The circadian amplitudes of *GI* mRNA abundance, similar to those of *CO*, were lower in *nox1* than WT (Fig. 4, C and D), which suggests that NO acts upstream of CO. Additionally, we analyzed the flowering phenotypes of *elf3* and *CO* overexpressing plants (*35S::CO*) (*22*) in response to NO. NO suppression of flowering was largely abolished in *elf3* mutants and *35S::CO* plants (Fig. 4F), which further supports the role of NO in the photoperiod pathway.

The expression of *CO* is regulated by the circadian clock and photoperiod, and the circadian rhythm of *CAB* expression can represent the circadian output in plants (*2*). The amplitudes of *CAB* mRNA abundance after switching from 12-hour light/12-hour dark cycles to continuous light for 3 days were greatly reduced in *nox1*, as well as in *cue1* mutants (*13*), whereas the circadian phases were not altered (*8*), similar to the expression of *CO* and *GI*. The amplitudes of another circadian output, cotyledon leaf movements, were also reduced by 43.8 ± 4.9% in *nox1* compared with WT, and no alteration of the circadian period was observed (Fig. 4E; fig S7). To analyze whether NO affects the circadian clock itself, we examined components of circadian clock central oscillators, *TIMING OF CAB EXPRESSION* (*TOC1*) and *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) (*23*, *24*). Neither the amplitude nor the circadian period of *TOC1* and *CCA1* expression was altered in *nox1* (*8*). Therefore, it is more likely that NO may affect circadian outputs rather than these central oscillators. Additionally, NO content in leaves in the middle of the night was 70.6% of that in the middle of the day in a 12-hour light/12-hour dark cycle for WT plants and 58.0% for *nox1* (table S2). This result suggests that the endogenous NO levels display a diurnal rhythm, consistent with a previous study (*7*). Although it is unclear whether NO production is dependent on light or the circadian clock, the alteration of NO homeostasis in *nox1* may cause its flowering phenotype.

We have provided pharmacological, physiological, molecular, and genetic data demonstrating that NO represses the photoperiod and autonomous floral pathways. These results are largely consistent with previous observations of delayed development in tobacco where NO is overproduced and of its inhibitory effects on maturation and senescence (*5*, *7*). Given that NO is induced by environmental changes, as well as constitutively produced, external and internal cues may converge on the regulation of endogenous NO status, which then relays these signals to the transcription regulatory network that controls the floral transition, which provides a unique layer of floral regulation. Oscillating NO levels, in addition to *CCA1* and *TOC*, may provide a mechanism to measure day/night switches, acting downstream of light perception to regulate certain circadian outputs and thus to control the floral transition, although the precise action of NO remains to be determined. Because several genes affected by NO (*CAB*, *CO*, and *GI*) are also regulated by light, the primary effect of NO may be on light signaling, and the effect on flowering may be secondary. Thus, identification of the direct target of NO will further our understanding of the molecular function of NO in plants.

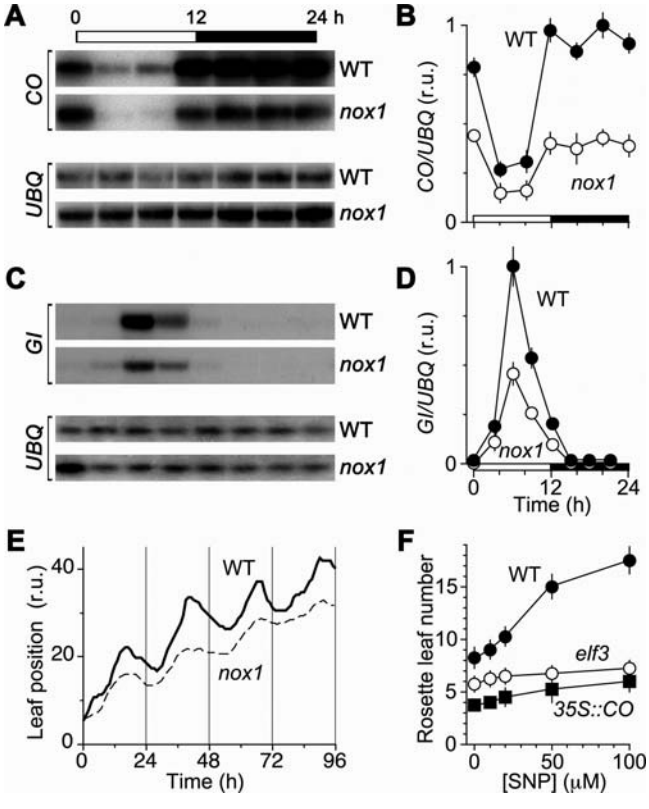

Fig. 4. NO suppresses the photoperiod pathway. (**A** and **C**) The mRNA abundance of *CO* and *GI* over a 24-hour time course. Plants were grown under 12-hour light/12-hour dark cycles for 10 days. Seedlings were collected every 4 hours for *CO* and every 3 hours for *GI* starting at dawn. The black and white bars at the top represent objective lights off and on, respectively. (**B** and **D**) Quantification of *CO* and *GI* mRNA abundance in (A) and (C), respectively (mean ± SEM; $n = 5$). (**E**) Circadian rhythms of cotyledon movements. Seedlings were grown under 12-hour light/12-hour dark cycles for 5 days, and cotyledon movements were recorded under constant dim light (*10*). (**F**) Analysis of the flowering time of *elf3* and *35S::CO* in response to SNP treatments (mean ± SD; $n = 150$ seedlings).

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Supporting Online Material

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